Abstract  *Hizikia fusiforme* is a commonly used food that possesses potent anti-bacterial, anti-fungal, and anti-inflammatory activities. The immunostimulatory activities of aqueous extract of *Hizikia fusiforme* (HFAE) in RAW 264.7 macrophages and whole spleen cells were investigated. HFAE activated RAW 264.7 macrophages to produce cytokines such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in a dose-dependent manner. In addition, HFAE induced the mRNA expression of TNF-α, IL-1β, and IL-6 in RAW 264.7 macrophages. Moreover, HFAE stimulated proliferation of whole spleen cells and reference mitogen. Taken together, the results demonstrate that HFAE potently activates the immune function by regulating NO, TNF-α, IL-1β, and IL-6 in RAW 264.7 macrophage and promoting spleen cell proliferation.

Keywords: *Hizikia fusiforme*, immunostimulatory activity, nitric oxide, RAW 264.7 macrophage, spleen cells

1. Introduction

*Hizikia fusiforme* is a brown seaweed that mainly grows in the temperate seaside areas of the northwest Pacific, including China, Japan [1], and Korea, and which has been commonly used as a health food in Korea. *H. fusiforme* contain fucoids, polysaccharides that contain a number of fucose and sulfate ester groups. Many plant-derived polysaccharides have been studied because of their immunostimulating activity. The enhancement of host immune responses has been recognized as a possible means for inhibiting tumor growth without harming the host, and extensive studies have been undertaken to find immunostimulatory biomaterials from a variety of sources [2]. Some immunocompetent herbal extracts have also been reported, including the aqueous extract of *Mori Fructus*, aqueous extracts and polysaccharides of *Panax ginseng*, *Lentinus edodes*, *Angelica gigas*, and *Pellinus linteus* [2-5]. Polysaccharides isolated from plants and algae have been reported to enhance macrophage activation [6-8]. A variety of biological activities, including anti-cancer [9] and anti-inflammatory [10] activity of *H. fusiforme* have been reported, but little is known about the molecular mechanism of *H. fusiforme* as an immunostimulator.

Macrophages and lymphocytes are representative immune cells that regulate innate and adaptive immunity of the host defense. Macrophages are the first cells to recognize invad-
ing foreign bodies and are central to cell-mediated and humoral immunity [11]. Stimulation of macrophages by lipopolysaccharide (LPS) enhances the production of inflammatory mediators, such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) [2]. LPS is a key component of the cell walls of Gram-negative bacteria, and triggers the activation of monocytes and macrophages involved in infection response [12]. NO, which is an inflammatory mediator, is produced by the activation of inducible nitric oxide synthase (iNOS) from L-arginine [13]. The expression of iNOS in activated macrophages is responsible for the profound production of NO that acts to kill tumor cells and pathogens [14]. Cytokines are significant in the regulation of the inflammatory and immune responses [15].

The spleen is a critical immune organ that contains lymphocytes and other immune cells. The spleen serves as the major site for recognition of blood-borne antigens by the immune system and is involved in the removal of old or damages in blood.

In the present study, we investigated the immunostimulatory activity of aqueous extract of *H. fusiforme* (HFAE) in RAW 264.7 macrophages and whole spleen cells of C57/BL6 mice.

2. Materials and Methods

2.1. Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. LPS was from *Salmonella typhosa* (Sigma-Aldrich). Fetal bovine serum was from Gibco BRL Life Technology (Rockville, MD). HEAE was supplied by Wando-gun, Jellannam-do, South Korea. HEAE was prepared as follows. Distilled water at 70°C was added to dry *H. fusiforme* and the temperature was maintained for 5 h. The mixture was allowed to cool to room temperature, filtered, and lyophilized. The lyophilized extract powder was dissolved directly in distilled water.

2.2. Animals and cell culture

C57/BL6 mice were obtained from Daehan Biolink (Chungbuk, Korea) and were maintained under specific pathogen free conditions until used. RAW 264.7 macrophages (ATCC TIB71) were purchased from American Type Culture Collection (Bethesda, MD, USA). Cells were grown in DMEM (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gibco BRL), 100 units/mL penicillin, and 100 µg/mL streptomycin. Non-adherent cells were removed by repeated washing after 2 h incubation at 37°C. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

2.3. Cell viability assay

Cell viability was evaluated by a 3-(4,5-dimethylthiazol-1)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTS) assay [16]. In the MTS assay, 100 µL aliquots of the cell suspension were plated in a 96-well microculture plate. After seeding, various concentrations of the compound were added to the plate and incubated for 24 h. A solution of MTS and phenazinemethosulfate (PMS) solution (1.53 mg/mL in PBS) was prepared by mixing 25 µL PMS for every 975 µL MTS (1.71 mg/mL in PBS). Finally, 50 µL of the MTS/PMS solution was added to each well and incubated for 1 ~ 3 h. The absorbance of formazan at 490 nm was measured directly from the 96-well assay plates without additional processing.

2.4. Spleen cell proliferation assay

Spleens were removed aseptically and homogenized in sterile Hanks’ balanced salt solution (Sigma-Aldrich). Splenocytes were sedimented by centrifugation, resuspended in red blood cell lysing buffer (Sigma-Aldrich) for 10 min, washed, and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 µM 2-mercaptoethanol, penicillin G (100 U/mL), streptomycin (100 µg/mL), 1 mM sodium pyruvate, and 2 mM L-glutamine (Sigma-Aldrich). Cell suspensions were distributed (1 × 10⁶ cells/mL) viable cells per well into 96-well tissue culture clusters with flat-bottom wells (Costar, Cambridge, MA, USA). Specific lymphocyte mitogens, such as LPS, were used as reference at a final concentration of 1 µg/mL. Cells were pulsed with 1 µCi/well of ³²H-thymidine (113 Ci/nmol; NEN, Boston, MA, USA) for the last 18 h of the 3-day incubation and harvested with an automated cell harvester (Inotech, Dottikon, Switzerland). The amount of ³²H-thymidine incorporated into the cells was measured using a Microbeta scintillation counter (Wallac, Turku, Finland).

2.5. Nitrite quantification

Nitrite accumulation was used as an indicator of NO production in the medium as previously described [17,18]. Cells were plated at 5 × 10⁵ cells/mL in 96-well culture plates and stimulated with HFAE or LPS for 24 h. The isolated supernatants in cultured macrophages were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Using NaNO₂ to generate a standard curve, the concentration of nitrite was measured by the optical density at 540 nm.